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THE STUDY OF THE SPOROCYST BROODSACS COLORING IN *LEUCOCHLORIDIUM PARADOXUM* (TREMATODA: BRACHYLAEMIDAE)

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The secretory cells were found in the subtegument of the sporocysts *Leucochloridium paradoxum* by histological assay. Pigment granules are formed by these cells. The movement of granules from secretory cells to the tegument external layer was observed. These pigment granules provide the yellow color of sporocysts broodsacs and the brown color of protuberant spots in the terminal part of broodsacs. It was shown that the pigment granules did not contain proteins, nucleotides, lipids and carbohydrates. The positive result was received while staining on bile pigments. The question on the nature of the green pigment remains open. The paletot on the surface of sporocyst formed by spreading hemocytes was observed. This structure was not described before in brachylaemid parthenites.

Key worlds: sporocysts, *Leucochloridium paradoxum*, pigment granules, broodsac, tegument, paletot.

ИЗУЧЕНИЕ ОКРАСКИ ОТРОСТКОВ СПОРОЦИСТ *LEUCOCHLORIDIUM PARADOXUM* (TREMATODA: BRACHYLAEMIDAE)

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В спороцистах *Leucochloridium paradoxum* обнаружены секреторные клетки, в которых формируются пигментные гранулы, прослежены пути их перемещения к тегументу. Именно эти пигменты окрашивают отростки спороцист в желтый, а бугорки в терминальной части отростков — в коричневый цвет. Установлено, что в пигментных гранулах отсутствуют белки, липиды и углеводы. Получен положительный результат при окрашивании на желчные пигменты. Вопрос о природе зеленого пигмента остается открытым. На поверхности спороцисты имеется мантия, образованная распластанными гемоцитами. Ранее эта структура у партенит Brachylaemidae не описывалась.

Ключевые слова: спороцисты *Leucochloridium paradoxum*, пигментные гранулы, отростки, тегумент, мантия.

The main taxonomic characters for species identification in the genus *Leucochloridium* Carus, 1835 include the shape and coloring of mature broodsacs of sporocysts, sprouting into the feelers of molluscs (Ginetsinskaya, 1953, 1968; Pojmanska, 1967; Bakke, 1980, etc.). There are several classifications based of broodsac coloring. Firstly, four main pigmentation characters, namely, the presence of green, orange, russet, and brown sporocysts were distinguished by Woodhead (1935). Later, the genus *Leucochloridium* was subdivided into five species according to broodsac coloration: green sporocysts (the description of coloring agrees with the description carried out by Carus (1835) for *Leucochloridium paradoxum*); orange sporocysts (agrees with Woodhead's description); yellow-brown sporocysts; brown-green sporocysts, and warty sporocysts (*L. vogtianum*) (Ginetsinskaya, 1953, 1968). Nacheva et al. (1981) mentioned only three types of sporocysts in the genus *Leucochloridium*: green sporocysts in *L. paradoxum*, brown sporocysts in *L. problematicum*, and brown-green sporocysts (the species was not identified). In the recent publications, the objectivity of taxonomic criteria based on coloring of broodsacs was confirmed by molecular genetic methods (Casey et al., 2003; Rzad et al., 2011; Zhukova et al., 2012).

However, the nature of broodsac coloring remains unclear. It is known that the intensity of band coloring and spots on the surface of the sporocyst broodsac depends on the density of distribution of pigment granules. In those parts of the tegument, where the density of granules is low, the arrangement of bands is more even and the coloring is less intense. The quantity of granules depends on the maturity of the sporocyst broodsac (Pojmanska, 1967).

The literary data on the chemical nature of *Leucochloridium* pigment granules are poor. In the present study, we used histochemical and scanning electron microscopy (SEM) methods to examine chemical nature and distribution of pigment granules in broodsacs of *Leucochloridium paradoxum* (Carus, 1835).

MATERIAL AND METHODS

The snail *Succinea putris* L., 1758 (Gastropoda: Succineidae) is the host of *L. paradoxum*. Snails (n = 50) were collected in the Vyritca village (Leningrad Province, Russia) in April—August 2012. Sporocyst broodsacs were dissected and fixed either in Bouin's fixative for histology or in 10 % buffered formalin for histochemical assay.

For preparation of paraffin sections, the fixed material was washed in 2—3 changes of 70 % ethanol and placed in paraffin after dehydration, according to the standard technique. The sections 4—8 μm were stained in hematoxylin-eosin, toluidine blue, alcian blue, gallocianin according to Einarson, amido-black 10B, potassium ferricyanide according to Schmorl, an iodic reagent according to Stein and silver nitrate (Pirs, 1962).

For histochemical identification of lipids, the cryosections were prepared. For this purpose a material was fixed in the 4 % paraformaldehyde in 0.1 M PBS (pH 7.4). Then it was washed in 0.1 M PBS and incubated in solution of

sucrose of ascending concentration (5, 10, 20, 30 %). In 30 % solution samples were incubated overnight at 4 °C. The sections 15 μm of thickness were prepared using Leica CM-3050S.

Sporocysts were fixed in glutaraldehyde-osmic acid (4 °C, pH 7.4, 425 mOsn) and subjected to further preparation for SEM according to the standard procedures (microscope Zeiss EVO 40).

RESULTS

The SEM study of the outer integument demonstrated that surface of the central part of the sporocyst stolon was strongly folded (fig. 1, *B*). The surface of young broodsacs was less folded, and the tegument of the mature (colored) broodsacs was smooth (fig. 1, *A*). However, the clearly visible protuberant spots were situated in the terminal part of the mature broodsac; they correspond to brown spots, typical of sporocysts of *L. paradoxum*. The rings are also present on the broodsac surface, topographically corresponding to green bands (fig. 1, *C*).

A paletot, that was not described earlier in Brachylaemidae parthenitae, was found on the surface of the sporocyst (fig. 1, *B*). In the central part of the stolon, the paletot is formed by molluscan haemocytes, spread on the surface of the parasite. The nuclei and pseudopodia of haemocytes are well noticeable. In this region, the paletot is loose and strongly perforated. On the surface of broodsacs, the paletot forms a continuous thin layer and nuclei of haemocytes are not visible on SEM micrographs (fig. 1, *A*). However they are well visible in histological sections (fig. 1, *D*).

The study of histological samples (fig. 1, *A*) has shown that the cells responsible for yellow-brown pigment granules secretion were located in the subtegumental layer of broodsacs. The highest concentration of pigment granules was observed in cytoplasmic projections of the secretory cells, which were directed towards the outer layer of the tegument. These cells were located around brown protuberant spots in the terminal part of the sporocyst broodsacs (see above). The movement of formed rounded granules (1—1.5 μm) to the tegument was well observed on histological sections. It is noticeable, that the concentration of the granules gradually increased toward the surface. This pigment provides yellow coloring of the surface of sporocyst broodsacs. Especially large accumulations of granules were noted in protuberant spots. That is the reason of their brown coloring and relief.

The following results were obtained during the histochemical study.

1) Pigment granules retained their color after staining with sudan black according to Lison (for identification of lipids). Only subtegumental structures of sporocyst (fig. 2, *B*) were stained.

2) Toluidine blue was used for detection of carbohydrates. Pigment granules also kept the intact color after staining (fig. 2, *C*).

3) Staining by amido-black was applied for identification of proteins. Pigment granules were not stained (fig. 2, *D*).

4) In order to verify the assumption about the bilious nature of pigment granules, sections were stained by potassium ferricyanide according to Schmorl. Positive reaction was observed (fig. 2, *E*).

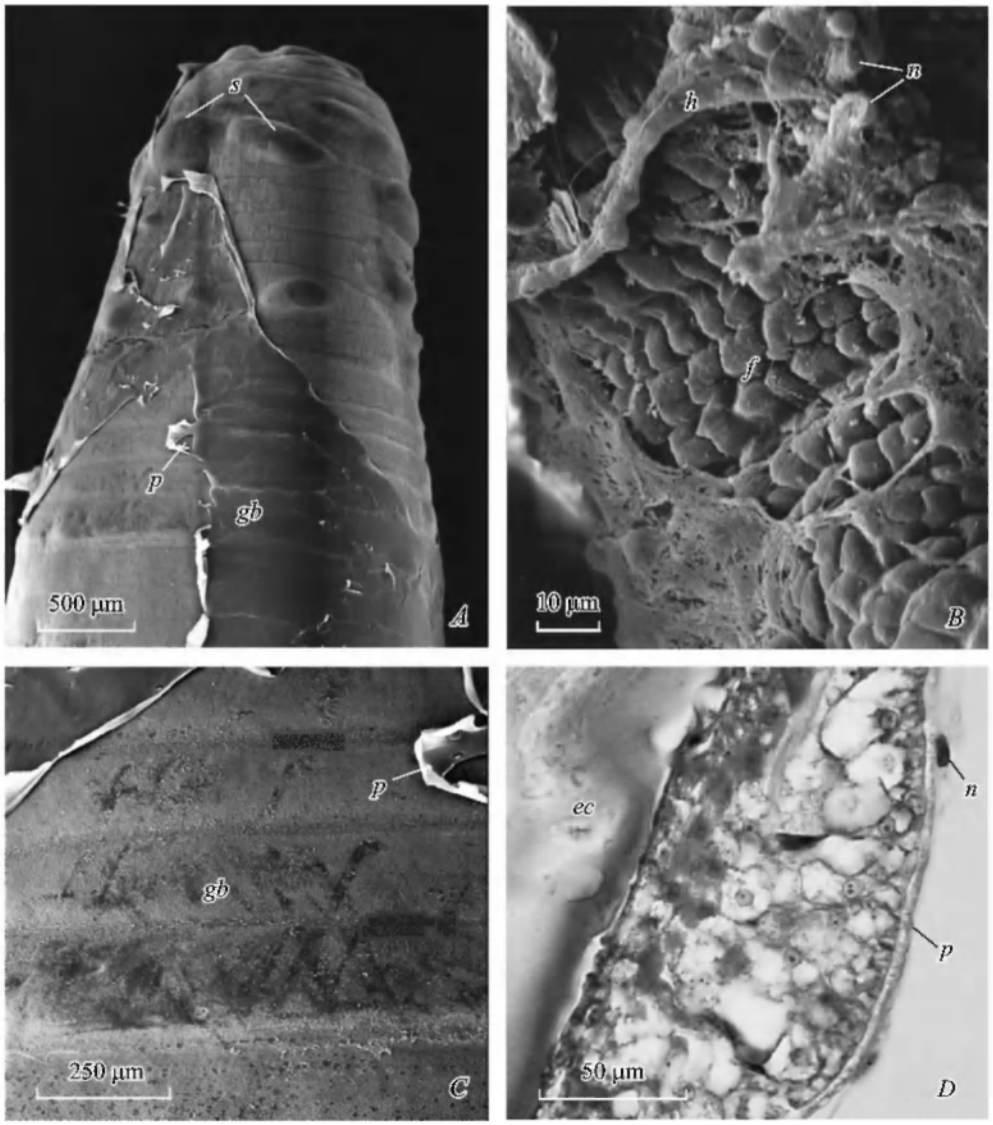

Fig. 1. SEM micrograph of *Leucochloridium paradoxum* sporocysts (*A*—*C*).

A — surface of sporocyst broodsac; *B* — surface of central part of stolon; *C* — surface of broodsac in green band region; *D* — histological section of broodsac wall. *ec* — embryonic cavity, *f* — folds of tegument, *gb* — green band, *h* — spreading mollusc haemocytes (paletot), *n* — nucleus of haemocyte, *p* — paletot, *s* — protuberant spots.

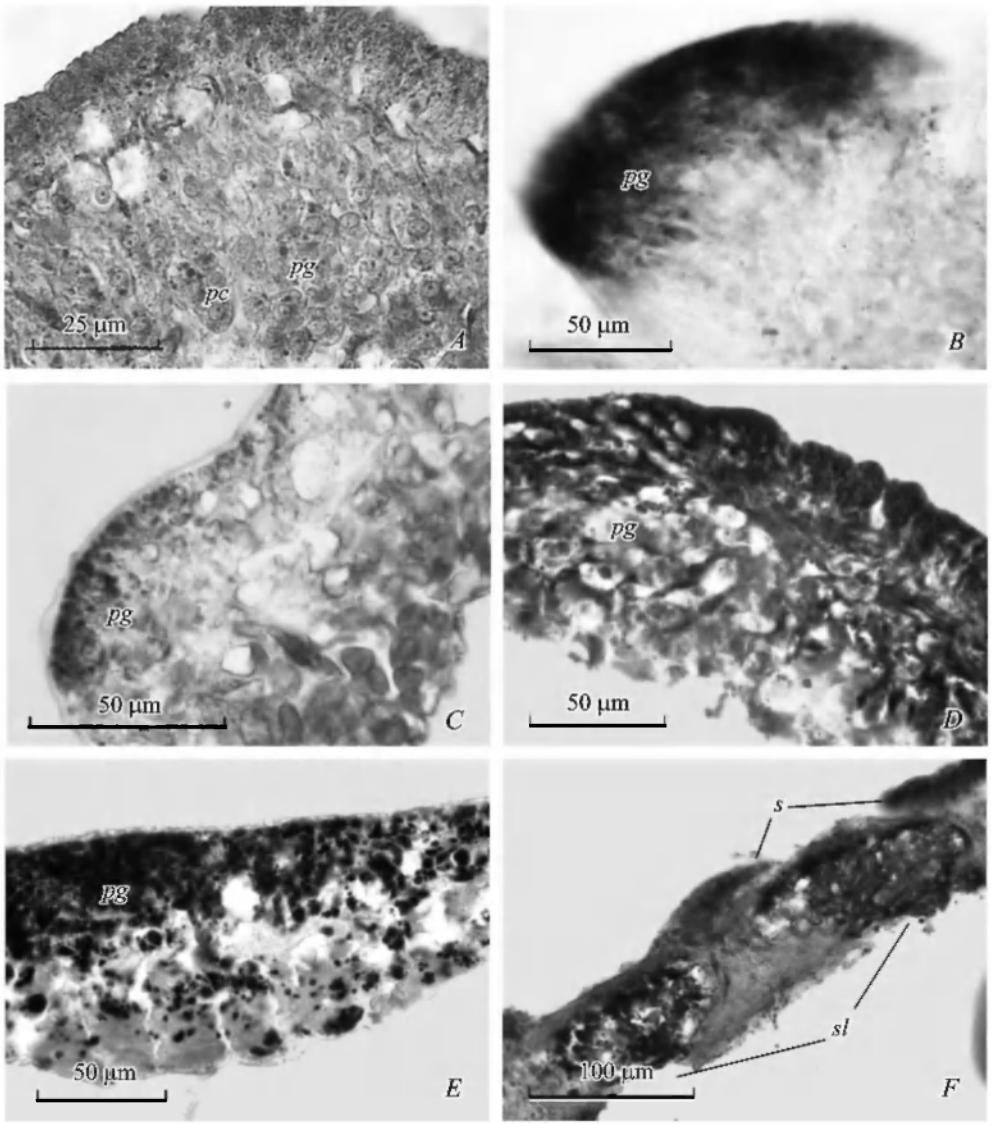

Fig. 2. Histological sections of broodsac body wall of *Leucochloridium paradoxum* sporocysts (*A*—*C*).

A — staining with hematoxylin-eosin; *B* — staining with sudan black according to Lison (for identification of lipids); *C* — staining with toluidine (for detection of carbohydrates); *D* — after the staining by amido-black (for identification of proteins); *E* — staining with potassium ferricyanide according to Schmorl (for the vertification of the assumption on the bilious nature of pigment granules; positive reaction); *F* — staining according to Glenner's three-color method (for identification of bilirubin, hemosiderin and lipofuscin). *pc* — pigment cells, *pg* — pigment granules, *s* — protuberant spots, *sl* — subtegumental layer.

5) After staining with the iodine reagent according to Stein (for identification of lipofuscin), the color of pigment granules also did not change. However, staining with the three-color method, which reveals bilirubin, hemosiderin and lipofuscin, demonstrated that the latter substance was present in the subtegumental layer between protuberant spots (fig. 2, *F*).

6) The color of granules was not changed after staining of sections according to Felgen and with gallocianin according to Einarson (for identification of nucleic acids).

The green pigment was not stable in ethanol and Buen's reagent. Its distribution was observed on cryosections and after fixing with formalin. This pigment was present as irregular shape lumps with the size about 3 μm.

DISCUSSION

Studying of the sporocyst tegument confirmed the validity of subdivision of *L. paradoxum* sporocyst body into the zones distinguished earlier (Bakke, 1982; Pojmanska, Mahaj, 1991; Ataev et al., 2013): 1) the central part of the stolon, 2) the system of tubules, 3) broodsacs. Embryos of metacercariae are formed in the central zone, wherefrom they enter broodsacs through the tubules, where they mature. Therefore, the central part of sporocyst is reproductive and the colored sacs play the role of brood chambers. It is important to note that the central part is not only the reproductive, but, obviously, the trophic part of the stolon. In this region, the surface of the tegument is distinctly folded (by contrast to the smooth tegument of other sites). The same conclusion was made by other researchers basing on TEM data (Storch, Welsch, 1970; Bakke, 1982; Pojmanska, Mahaj, 1991). They have revealed the presence of numerous microvilli on the surface of the central part of the stolon.

The obtained data confirmed the results of other authors (Bakke, 1982; Zdarska et al., 1982), who had found yellow-brown pigment granules in the sporocyst tegument of *L. paradoxum* and had also described secretory cells. Storch and Welsch (1970) assumed participation of microtubules in the delivery of pigment granules in the external layer of secretory cells cytoplasm. Bakke (1982), however, did not manage to find specialized cytoskeleton structures in pigment cells.

We also managed to find secretory cells in histological sections and to trace transition patterns of pigment granules to the tegumental external layer. These pigments provide the yellow coloration of broodsacs and the brown color of protuberant spots in the terminal part. It was shown that pigment granules did not contain proteins, nucleotides, lipids and carbohydrates. The positive result was received while staining for bile pigments.

The question concerning the nature of the green pigment remains open. It is known that large lumps located in the tegument in the region of green bands, provide this coloring. However, the source of this pigment as well as its chemical nature is not clear. There is only Nacheva's et al. (1981) assumption that yellow-brown granules can merge and form large lumps. Indirect confirmation of this assumption can be found in the size of green lumps that are twice as large as yellow-brown pigment granules, and also in the relative instability of this green pigment.

An original hypothesis has been assumed by Nacheva et al. (1983). According to this hypothesis, the distribution of granules is caused by reciprocal adaptation of the parasite and the host. The sporocyst gets nutrients from the molluscan digestive gland and affects its nutrition. By contrast, the digestive gland partly adapts to the presence of the parasite and consumes nutrition from the sporocyst body wall. In the opinion of Nacheva et al. (1983), the latter process explains accumulation of different types of pigment granules in digestive glands of a molluscan species depending on *Leucochloridium* sporocyst parasitizing in it. Unfortunately, no empirical data supporting the authors' hypothesis is mentioned in the aforementioned article.

According to other authors (Mönnig, 1922; Wesenberg-Lund, 1931; Pojmanska, 1967; Bakke, 1982), the pigmentation of sporocyst broodsacs of *Leucochloridium* appears only under the influence of the daylight, after they get into transparent mollusc feelers. It is also supposed that pigment cells play the important role in physiological reactions of sporocysts, for example, in broodsac pulsations (Lewis, 1977). Our data did not confirm these conclusions. Moreover, we observed active pulsations of colorless broodsacs.

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